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Distribution of ^{15}N within Pea, Lupin, and Soybean Nodules[1]

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ABSTRACT

The ^{15}N abundance of some, but not all, legume root nodules is significantly elevated compared to that of the whole plant. It seems probable that differences in ^{15}N enrichment reflect differences in the assimilatory pathway of fixed N. In that context, we have determined the distribution of naturally occurring ^{15}N in structural fractions of nodules from soybean (*Glycine max* L. Merr.), yellow lupin (*Lupinus luteus*), and pea (*Pisum sativum*) nodules and in chemical components from soybean nodules and to a lesser extent, pea and lupin nodules. None of the fractions of pea nodules (cortex, bacteriod, or host plant cytoplasm) was enriched in ^{15}N. The differences among bacteriods, cortex, and plant cytoplasm were smaller in lupin than in soybean nodules, but in both, bacteriods had the highest ^{15}N enrichment. In soybean nodules, the ^{15}N abundance of bacteriods and cortex was higher than plant cytoplasm, but all three fractions were more enriched in ^{15}N than the entire plant. Plant cytoplasm from soybean nodules was fractionated into protein-rich material, nonprotein alcohol precipitable material (NA), and a low molecular weight fraction. The N of the latter was further separated into N of ureides, nucleotides and free amino acids. Most of these components were either similar to or lower in ^{15}N abundance than the plant cytoplasm as a whole, but the NA fraction showed unusual ^{15}N enrichment. However, the percentage of nodule N in this fraction was small. NA fractions from yellow lupin and pea nodules and from soybean leaves were not enriched in ^{15}N. Nor was the NA fraction in ruptured bacteriods and cortical tissue of soybean nodules. Variation among soybean nodule fractions in the preponderance in protein of different amino acids was not large enough to explain the differences in ^{15}N abundances among them. A hypothesis, consistent with all known data, concerning the mechanism leading to the observed excess ^{15}N of lupin and soybean bacteriods is offered.

Nodules of certain, but not all, N_2-fixing plants have a substantially higher ^{15}N abundance than that of nonnodular tissues (7, 15, 18, 19), while the ^{15}N abundances of nonnodular tissues are usually similar to each other in ^{15}N abundance. When atmospheric N_2 is the sole source of N to the N_2-fixing plant, the $\delta^{15}N$ value[2] of the entire plant is usually within 2‰ of that of atmospheric N_2 (16). Ineffective soybean nodules are not enriched in ^{15}N, and the degree of enrichment is positively correlated with the quantity of N_2 fixed per quantity of nodule N (7, 8). It therefore seems probable that the unique ^{15}N enrichment of nodules is caused by isotopic fractionation associated with assimilation of fixed N, and that differences in ^{15}N enrichment among nodules of different kinds reflect differences in assimilatory pathways.

^{15}N enrichment of nodules is common to all ureide-exporting nodules so far examined (15, 18, 19, 21). This led to the hypothesis that ^{15}N enrichment of nodules was caused by isotopic fractionation associated with ureide synthesis (15). However, since that hypothesis was put forward, it has been shown that amide-producing nodules are also occasionally enriched in ^{15}N (1, 18, 19, 21, and our unpublished results).

Previous studies indicate that ^{15}N enrichment of nodules is concentrated in bacteroids (11, 19, 21) and cortical material (11). In this paper, we report results of further fractionation of nodules into structural and chemical components. We compare ^{15}N abundance of nodule fractions of ureide-exporting nodules (from soybean), of amide-exporting nodules which are enriched in ^{15}N (from lupin), and of amide-exporting nodules which are not enriched in ^{15}N (from peas).

METHODS AND MATERIALS

Plant Culture

Soybean (*Glycine max*) seeds (variety, Harosoy) were obtained from J. E. Harper (USDA-SEA, ARS, University of Illinois, Urbana), pea (*Pisum sativum*) seeds (Alaska) from the W-6 Regional Plant Introduction Station, USDA, Pullman, WA, and yellow lupin (*Lupinus luteus*) seeds (plant introduction No. 384565) from the Southern Regional Plant Introduction Station, USDA, Experiment, GA. Rhizobial inocula were obtained from the Nitragin Division of Lipha Chemicals, Milwaukee, WI. Soybeans were inoculated with Nitragin strain 61A76; yellow lupin with a commerical mix of strains 96B9, 96B15, 96A5, 96E3 and 96E7; and peas with strain 128C56. All inocula were provided stabilized on peat. After inoculation, seeds were planted in 8 inch pots (5 plants per pot) filled with perlite. Each kind of plant was grown in 40 pots for each experiment. Plants were fertilized with N-free nutrients according to the method described by Fishbeck (4). After germination, plants were thinned to 1 per pot (soybeans), or 3 per pot (peas and lupins). Four pots were used for quadruplicate samples of leaves, stems, reproductive tissues, roots, and nodules. These tissues were weighed and dried at 55°C for approximately 1 week. After drying, non-

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[2] $$\delta^{15}N = \frac{\text{atom\%}^{15}N(\text{sample}) - \text{atom\%}^{15}N(\text{standard})}{\text{atom\%}^{15}N(\text{standard})} 1000‰$$

where the standard is customarily atmospheric N_2.

nodular tissues were ground in a Wiley mill to pass a 20-mesh screen. Dried nodules were pulverized in a mortar and pestle. Dried tissues were analyzed for ^{15}N abundance and percent N. The ^{15}N abundance of the entire plant was calculated from the weighted average of leaves, stems, roots, reproductive tissues and nodules. The remainder of the pots were used for fractionation studies. Nodules were separated from roots. Soybean leaf tissue and nodules were weighed and held at −20°C until analyzed.

Fractionation of Frozen Nodules

Nodules were crushed in a mortar and pestle with 2.5 volumes of a grinding medium consisting of buffer (either 100 mM $NaHCO_3$ buffer [pH 9] or 100 mM potassium phosphate buffer, [pH7] with 1% sodium lauryl sulfate and 150 mM NaCl. The resulting slurry was filtered through four layers of cheesecloth. The nonfilterable material was reground in a second 2.5 volumes of grinding medium and filtered. The material remaining on the cheesecloth consisted chiefly of cortical and cell wall material, which was homogenized with a ground glass tissue grinder. The material passing through the cheesecloth consisted of bacteroids and plant cytoplasm from both infected and noninfected cells. Bacteroids were separated from plant cytoplasm by centrifugation for 10 min at 10,000*g*. The pellet was resuspended in grinding buffer. To this point, three major fractions were obtained (13, 17): a cortex-rich fraction (designated C), a bacteroid-rich fraction (designated B), and host plant cytoplasm (designated H). The N content and ^{15}N abundance of these major fractions were determined prior to further fractionation. Fraction B was disrupted in a French Pressure cell (15,000 p.s.i.). Particulate material was removed from fractions B and C by centrifugation at 10,000*g*.

Fraction H and the soluble portions of fractions B and C were further fractionated into protein-rich, nucleic acid-rich, and low mol wt components as follows. The samples were extracted three times with a volume of 1:1 (v/v) chloroform/phenol equal to the aqueous volume of the preparation in order to separate protein from nucleic acids and low mol wt components (10). The organic phase and interface were collected and dried by rotoevaporation at 70°C. The dried material was resuspended in H_2O and held for analysis. This fraction was designated the protein-rich fraction (P^3). The aqueous phase was diluted with 2.5 volumes of cold 95% ethanol and held overnight at −20°C. Precipitated material was recovered by centrifuging 20 min at 10,000*g*. The resultant pellet, designated as NA, was dissolved in H_2O and held for analysis. The ethanol supernatant was dried by rotoevaporation at room temperature and resuspended either in 10% $HClO_4$ or H_2O, as indicated. This material was designated LMW. At this point, measurements were made of the ^{15}N abundance and N content of all P, NA, and LMW fractions.

Nucleotides, urea (derived from ureides) and free amino acids were recovered from the low mol wt fractions as follows. The dried LMW fractions were dissolved in 10% $HClO_4$. This treatment results in quantitative hydrolysis of ureides to urea (20). The sample was then neutralized with KOH, and the insoluble $KClO_4$ was removed by low speed centrifugation. Nucleotides were adsorbed on activated charcoal (10 mg charcoal/mL sample) and separated from the sample by centrifugation (5). The supernate, containing urea and free amino acids, was adjusted to pH 2.2 with citric acid and 5 to 10 mL was loaded onto a 4 mL bed volume column of the Na^+ form[4] of Dowex 50 × 4-200 (Sigma, St. Louis MO 63178-9974). Urea, which does not bind to the column at pH 2.2, was eluted with 15 mL 200 mM citrate (pH 2.2). Free amino acids were eluted with 15 mL 2 M sodium citrate, acidified to pH 5.1 with acetic acid.

In some experiments a protein-rich fraction was prepared by alcohol precipitation (2.5 volumes cold ethanol to 1 volume sample). These fractions, which also contained nucleic acids and other alcohol precipitable materials were digested in 6 N HCl for determining amino acid composition of proteins in the major fractions (H, B, and C). These analyses were done by the Protein Chemistry Facility at Washington University Medical School on a Waters programmable HPLC equipped with *o*-phthalaldehyde detection and continuous infusion of $HClO_3$ for detection of secondary amines. The crude protein from the plant cytoplasm fraction prepared in this way contained a significant contaminant which ran with methionine, as discussed in "Results" below. Therefore, amino acid composition of proteins in the H fraction was determined on a preparation extracted in chloroform/phenol.

Nitrogen and ^{15}N Measurements

The quantity of nitrogen was measured by Kjeldahl digestion followed by steam distillation. After conversion of the N to NH_4^+, and evacuation to remove atmospheric N_2, N_2 was generated from the sample N by reaction with NaOBr. Nitrogen isotope composition of N_2 derived from the sample was measured with an isotope ratio mass spectrometer (VG Micromass 602E). Methods of sample preparation and isotope abundance determinations are described in detail elsewhere (16).

RESULTS AND DISCUSSION

Distribution of ^{15}N within Soybean Nodules at Different Growth Stages

Table I shows the distribution of ^{15}N in soybean nodules at three growth stages (V4, V6, and R7 as defined by Fehr *et al.* [3]). The ^{15}N abundance of the entire plant at these three growth stages was 0.8 ± 0.3‰ (V4), 0.1 ± 0.2‰ (V6), and 0 ± 0.3‰ (V7). As in previous studies, the ^{15}N enrichment of nodules increased with time during the growing season (7) and was concentrated primarily in bacteroids (11). The contribution of ^{15}N enrichment of bacteroids to total nodule enrichment peaked at midflowering (V6), while the contribution of cortical tissue to nodule ^{15}N enrichment was greatest

[3] Abbreviations: P, protein-rich fraction; NA, nucleic acid-rich fraction; LMW, low mol wt fraction.

[4] The Dowex 50 resin was prewashed with 4 N HCl until the filtrate was colorless. It was then washed with H_2O to remove the HCl, and finally it was washed with 2 N NaOH until the filtrate was alkaline. The column was equilibrated with 200 mM citrate (pH 2.2).

Table I. *Distribution of ^{15}N Abundance within Soybean Nodules from Plants at Three Stages of Development (V4, V6, R7 at 41, 53, and 90 d after Planting, Respectively)*

Days after Planting	Nodule Fraction	N Content	Nodule Nitrogen	^{15}N Abundance	^{15}N Excess in Nodule
		mg N/g nodule (fr wt)	%	*$\delta^{15}N$ (‰)*	%
41	Entire nodule	9.37 ± 0.55	100	6.1 ± 0.4	100
	Plant cytoplasm (H)	5.06 ± 0.73	54.0	4.1 ± 0.5	36.3
	Bacteroid (B)	0.47 ± 0	5.0	10.7 ± 0	8.8
	Cortex (C)	3.84 ± 0.13	41.0	8.2 ± 0.4	54.8
53	Entire nodule	9.48 ± 0.21	100	9.8 ± 0.4	100
	H	4.11 ± 0.21	43.1	5.7 ± 0.8	25.1
	B	3.64 ± 0.04	38.3	15.5 ± 0.8	60.7
	C	1.73 ± 0.02	18.2	7.5 ± 0.5	14.1
90	Entire nodule	8.36 ± 0.07	100	9.8 ± 0.1	100
	H	2.43 ± 0.03	29.1	4.5 ± 0.2	13.3
	B	3.57 ± 0.06	42.7	12.6 ± 0.1	54.9
	C	2.36 ± 0.01	28.2	11.1 ± 0.4	31.9

Table II. *Distribution of ^{15}N Abundance within Plant Cytoplasm of Soybean Nodules*

Days after Planting	Fraction[a]	N Content	Nodule Nitrogen	^{15}N Abundance	^{15}N Excess in Nodule
		mg N/g nodule (fr wt)	%	*$\delta^{15}N$ (‰)*	%
41	Plant cytoplasm	5.06 ± 0.73	54.0	2.2 ± 0.5	23.3
	HP	3.64 ± 0.73	38.8	1.5 ± 0.4	9.3
	HNA	0.85 ± 0	9.1	16.3 ± 0.5	24.2
	HLMW	0.57 ± 0	6.1	3.0 ± 0.5	2.4
	Ureides	0.35 ± 0		3.2 ± 0.8	
	Nucleotides	0.07		−0.5	
	Amino acids	0.15 ± 0		3.5 ± 0.1	
53	Plant cytoplasm	4.11 ± 0.21	43.5	5.7 ± 0.8	25.1
	HP	2.62 ± 0.20	28.0	2.1 ± 0.9	6.0
	HNA	0.79 ± 0.01	8.3	19.1 ± 0.2	16.3
	HLMW	0.70 ± 0.02	7.4	4.1 ± 0	3.1
	Ureides	0.51 ± 0		4.0 ± 0.2	
	Nucleotides	0.07 ± 0		0.9	
	Amino acids	0.08 ± 0		6.0 ± 0.3	
90	Plant cytoplasm	2.43 ± 0.03	29.1	4.5 ± 0.2	13.3
	HP	1.66 ± 0.03	19.9	3.6 ± 0.2	7.3
	HNA	0.26 ± 0	3.1	13.4 ± 0.2	4.2
	HLMW	0.51 ± 0	6.1	2.9 ± 0.1	1.8
	Ureides	0.41 ± 0		3.5 ± 0.1	
	Nucleotides	0.09 ± 0		1.1	
	Amino acids	0.02 ± 0		0.9	

[a] HP is the protein-rich subfraction of the host plant cytoplasm; HNA is the nucleic acid-rich subfraction of the host plant cytoplasm; and HLMW is the low mol wt subfraction of the host plant cytoplasm.

at the earliest harvest (V4). The contribution to total nodule enrichment of plant cytoplasm decreased during the growing season from ~36% at V4 to ~13% at R7.

Table II shows the result of further biochemical fractionation of the host cytoplasmic fraction of soybean nodules into a crude protein fraction (organic phase of a chloroform-phenol extract), a nucleic acid-rich fraction (alcohol precipitable portion of the aqueous phase of the chloroform-phenol extract), and various components (ureides, nucleotides, and free amino acids) of the low mol wt fraction (alcohol soluble portion of the aqueous phase of the chloroform-phenol extract). The most striking feature of this table is the very high and consistent ^{15}N enrichment of the nucleic acid-rich fraction. The contribution of this fraction to overall nodule ^{15}N enrichment declined with plant maturity from 24% at 41 d postplant to 3% at physiological maturity. Although this fraction is not a major contributor to overall ^{15}N enrichment of nodules, because the amount of N in this fraction is small (less than 10% of the total nodule N), it could contain ^{15}N enriched material in flux to some sink (*e.g.* bacteroids). It

should be noted that this crude fraction contains material other than nucleic acids. It would be surprising if nucleic acids themselves were as enriched in ^{15}N as the entire fraction in view of the low ^{15}N abundance of the free nucleotides. The decline for the entire 'plant cytoplasm' subfraction in the '% of ^{15}N excess in nodule' from 36.3 to 25.1 to 13.3% for nodules from 41-, 53-, and 90-d-old plants, respectively, is clearly due to the decreasing importance of the ^{15}N enrichment of HNA. At present, we offer no hypothesis concerning the biochemical or physiological significance of the unique ^{15}N enrichment of the HNA subfraction. This phenomenon is currently being investigated in detail.

The protein-rich fraction of the plant cytoplasm was consistently lower in ^{15}N than that of the entire fraction, as was the low mol wt fraction. The ^{15}N abundance of ureide N (3.2–4.0 ‰) was a few per mil higher than that of the N of the entire plant (0.1–0.8‰), suggesting a small isotopic fractionation associated with export of ureides from the nodule. This observation is in agreement with that reported by Yoneyama *et al.* (21) who also measured the ^{15}N abundance of ureides in the stem. They reported that, in soybeans, $\delta^{15}N$ values for nodule and stem ureides were 4.0 and 0.6‰, respectively. The small ^{15}N enrichment of nodule ureides cannot account for all of the overall enrichment of the nodule (6.1–9.8‰ in the experiments of Table I). Free amino acids were also only slightly enriched in ^{15}N, although these data cannot by themselves rule out that individual free amino acids which might be transported to bacteroids or cortex could be significantly enriched in ^{15}N (but see later discussion of Fig. 1). We offer no explanation for the dramatic decline in the ^{15}N abundance of the amino acid subfraction between the two later sampling dates (6.0 *versus* 0.9‰, respectively).

A similar, but less exhaustive, fractionation was carried out on the soluble portion of bacteroids (disrupted in a French pressure cell) and cortex (disrupted by glass homogenization) of soybean nodules harvested at 90 d after planting (Table III). Only a portion of the N in the intact bacteroid-rich and cortext-rich fractions was recovered in their soluble subfractions (69 and 47%, respectively). Both soluble subfractions were less enriched in ^{15}N than the fractions from which they were extracted (11.0 *versus* 12.6‰ for bacteroid-rich fractions and 7.8 *versus* 11.1‰ for cortex-rich fractions). Table III shows that most of the N of the soluble fractions, as well as most of the ^{15}N enrichment, was recovered in the protein-rich component (the organic phase and interface of a chloroform/phenol extract). The unusual enrichment of the crude nucleic acid fraction observed in the plant cytoplasm was not seen in bacteroids and cortex. The NA and LMW components were both less abundant in ^{15}N than the entire fraction.

Table III. *Distribution of N and ^{15}N within Bacteroids and Cortex of Soybean Nodules Harvested at 90 d after Planting (R7)*

Fraction	Total N in Fraction	^{15}N Abundance	^{15}N Excess in Fraction
	%	$\delta^{15}N$ (‰)	%
Total bacteroid-rich fraction		12.6 ± 0.1	
French-pressed bacteroids (soluble portion)	100	13.4 ± 0.5	100
Protein-rich fraction (BP)	78.2	13.4 ± 0.5	86.1
Nucleic acid-rich fraction (BNA)	18.1	6.4 ± 0.8	11.5
Low mol wt fraction (BLMW)	3.7	6.4	2.4
Total cortex-rich fraction		11.1 ± 0.4	
Glass homogenized cortex (soluble portion)	100	7.8 ± 0.5	100
CP	76.5	8.8 ± 0.1	87.4
CNA	11.1	5.2 ± 0.2	7.5
CLMW	12.3	3.3 ± 0.2	5.1

Comparison of the Distribution of ^{15}N Abundance within Pea, Lupin, and Soybean Nodules

Table IV shows the N content and ^{15}N abundance of the three major fractions (protein-rich, nucleic acid-rich, and low mol wt) of pea, yellow lupin, and soybean nodules, all harvested at midbloom. In this experiment, as in the experiment of Table I, the bacteroid fraction contributed most to the ^{15}N enrichment of soybean nodules at midbloom. In lupin nodules, the ^{15}N enrichment was more evenly distributed among the fractions. Although bacteroids had the highest $\delta^{15}N$ value of the three fractions, they accounted for only about 11% of the total N in the nodule. Pea nodules were not significantly enriched in ^{15}N, being within 1‰ of atmospheric N_2. Nor were any of the pea nodule fractions enriched in ^{15}N.

The H (plant cytoplasm) fractions of Table IV as well as the soluble fraction from soybean leaves collected from the same soybean plants were extracted with chloroform/phenol, and the aqueous phase was precipitated in ethanol (see "Materials and Methods") to produce a nucleic acid rich fraction in order to examine the generality of the high ^{15}N enrichment observed before (Table II). Table V shows that, of the tissues examined, this fraction is enriched only in soybean nodules (about 17‰). It is clear that ^{15}N enrichment of the nucleic acid-rich fraction is not required for overall nodule enrichment, since the lupin nodules were enriched, but the nucleic acid-rich fraction of lupin nodule plant cytoplasm was not. It would be of interest to determine whether this fraction is generally enriched in ureide-exporting nodules, as well as to identify the component of this crude fraction which carries the unusual enrichment. Such studies are presently underway.

Amino Acid Composition of Crude Protein Preparations from Soybean Nodules

It is by now clear that ureide synthesis is not required for ^{15}N enrichment of nodules, since certain amide-exporting nodules, when they arise from inoculation with an appropriate symbiont, are enriched in ^{15}N. Nevertheless, the question remains, does isotopic fractionation associated with ureide synthesis contribute to the enrichment of ureide exporting nodules? The results reported in this paper (Table II) and elsewhere (11, 19, 21) suggest that this is not the case since the host cytoplasm, where ureide synthesis occurs, is less

Table IV. *Comparison of Soybeans, Lupins, and Peas with Respect to Distribution of ^{15}N Abundance within Nodules*

Plant	Days after Planting	Nodule Fraction	N Content	Nodule Nitrogen	^{15}N Abundance	^{15}N Excess in Nodule
			mg N/g fr wt mean ± SE	%	*$\delta^{15}N$ (‰) mean ± SE*	%
Soybean	54	Entire nodule	10.90 ± 0.10	100	8.2 ± 0.5	100
		Plant cytoplasm (H)	6.24 ± 0.04	51.1	6.2 ± 0	27.5
		Bacteroid (B)	3.48 ± 0	28.5	13.2 ± 0.7	50.6
		Cortex (C)	2.52 ± 0.08	20.7	8.0 ± 0.1	22.2
Lupin	66	Entire nodule	12.10 ± 0.70	100	6.8 ± 0.2	100
		H	5.60 ± 0	48.7	5.7 ± 0	45.2
		B	1.31 ± 0.09	11.4	9.0 ± 0.8	16.6
		C	4.62 ± 0.02	40.2	5.9 ± 0.4	38.6
Pea	53	Entire nodule	9.30 ± 0.73	100	0.7 ± 0.2	
		H	2.62 ± 0.70	37.4	0.7 ± 0.4	
		B	1.55 ± 0.01	22.1	−1.6 ± 0.6	
		C	2.9 ± 0.6	40.9	−0.1 ± 0.4	

Table V. *Comparison of the ^{15}N Abundance of the Alcohol Precipitable Fraction of the Aqueous Phase of a Chloroform/Phenol Extract (Nucleic Acid-Rich Fraction) in Pea, Lupin, and Soybean Nodules and in Soybean Leaves*

Tissue	Total N in Nucleic Acid-rich Fraction	^{15}N Abundance	
		Total N	Nucleic acid-rich fraction
	%	*$\delta^{15}N$ (‰)*	
Pea nodule	1.14 ± 0	0.0 ± 0.4	−0.6
Lupin nodule	1.65 ± 0	6.1 ± 0.2	0.1 ± 0.8
Soybean nodule	6.00 ± 0	7.4 ± 0.3	17.1 ± 0.3
Soybean leaves	2.49 ± 0	−0.7 ± 0.8	−1.4

enriched in ^{15}N than any other fraction. The conclusion that isotopic fractionation associated with ureide synthesis does not contribute to ^{15}N enrichment of nodules would not be inevitable if ^{15}N abundances of the amino acids involved in ureide biosynthesis were both elevated and disproportionately represented in bacteroid and cortex protein (compared with host cytoplasm protein). The basis for this suggestion is most easily understood by considering three of the fates of glutamine, aspartate, and glycine in the host cytoplasm. The quantitatively most significant fate is assimilation into ureides (12). Since changes in the bonding to N occur, it is not unreasonable to postulate a normal isotope effect. This would result in the unused pool of these amino acids being enriched in ^{15}N. Part of this pool is used to synthesize host protein, while part might be exported to the bacteroids and cortex, to be used there to synthesize the proteins which are observed to be enriched in ^{15}N (Table III). However, in these circumstances, host cytoplasm proteins would be as enriched in ^{15}N as are proteins of bacteroid and cortex-rich fractions unless the mol percent of these amino acids is much higher in the latter two fractions than it is in the former. To examine this question we prepared crude protein fractions of soybean nodules by alcohol precipitation of the plant cytoplasm, the soluble portion of French-pressed bacteroids, and the soluble portion of the glass homogenized cortex-rich fraction. In the case of plant cytoplasm, it was necessary to partially purify the preparation by extraction with chloroform/phenol because of an interfering contaminant (discussed below) in the alcohol precipitate. The organic phase (and interface) was evaporated to dryness and extracted with hexane to remove lipids.

The interfering contaminant in the alcohol precipitate of the plant cytoplasm had the same retention time as methionine in hydrolyzed samples run on HPLC and subsequently derivatized with *o*-phthalaldehyde. The fluorescent intensity of this peak was so great that, had it been methionine, it would have accounted for up to 70% of the amino acids in the protein of the plant cytoplasm. Peracid oxidation to convert methionine to its sulfone resulted in separation of methionine sulfone from the presumed contaminant which remained in the methionine position. The contaminant contained a primary amine since $HClO_3$ treatment was not required to observe it. When the hydrolyzed sample was run with a program which separates glucosamine from methionine, the contaminant ran with glucosamine, but when run on a Dionex column (QIC chromatography system eluted with 0.15 N NaOH), the presumed contaminant did not run with glucosamine. It was not a constituent of a glycoprotein as indicated by electrophoresis on two SDS/polyacrylamide sizing gels, under reducing conditions. One of the gels was stained for protein (Coomassie blue), and the other was stained for amino sugars (periodic acid Schiff's reagent). As expected, the major protein band was at the position where one would anticipate finding leghemoglobin. Hexosamine staining material did not enter the dense gel, indicating either that the mol wt was high or that the molecules were not negatively charged. This material did not enter the organic phase or interface of a chloroform/phenol extract of the plant cytoplasm. Given the presence of the contaminant, the amino acid composition of proteins of the plant cytoplasm was determined on material extracted with chloroform and phenol.

Figure 1 shows the amino acid composition of proteins from plant cytosol (H), bacteroids (B), and the cortex-rich fraction (C). The mol percent of glutamate (the sum of glutamate plus glutamine residues), aspartate (the sum of aspartate plus asparagine residues) and glycine in the plant cytoplasm was 11.0, 10.0, and 7.9, respectively. For bacteroids, the comparable percentages were 9.3, 9.2, and 9.3, and

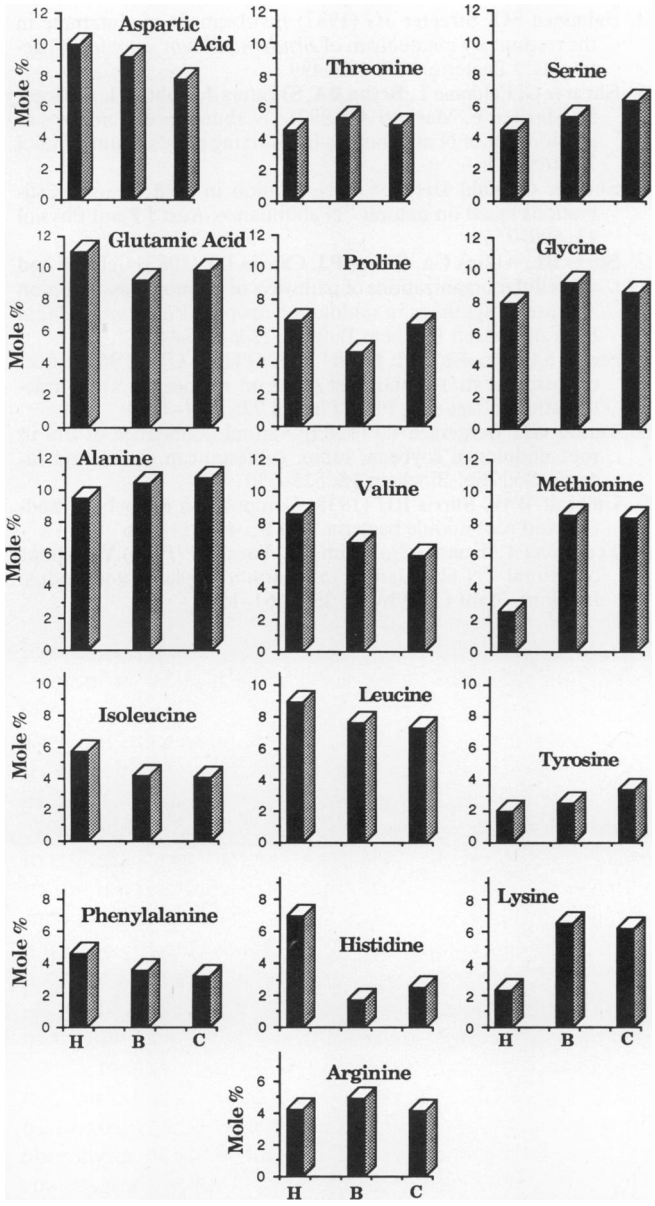

Figure 1. Abundance of amino acid residues in crude protein preparations in three major fractions of soybean nodules: Plant cytosol (H), bacteroids (B), and cortex (C). The H fraction was prepared by chloroform/phenol extraction. The B and C fractions contained the ethanol precipitable material of the nonparticulate portion of these fractions after disruption as described in "Materials and Methods."

for cortex, the percentages were 9.9, 7.8, and 8.6. Thus, the differences in abundance of the amino acids relevant to ureide synthesis were not large enough to account for the much higher natural abundances of ^{15}N in bacteroids and cortex than in the plant cytoplasm. Nor were there any differences between these fractions in the abundances of other amino acids large enough to cause the observed ^{15}N abundance differences. For example, histidine in the plant host cytoplasm fraction (H) would have to be 140‰ lighter than in the bacteroids to account for the 7‰ difference in ^{15}N abundance observed in those fractions (Table IV, row 2 *versus* row 3). This is beyond the realm of possibility.

It has recently been proposed that a significant fraction of the energy required for N_2-fixation is supplied to bacteroids from plant cells in the form of carbon skeletons of an amino acid (6). Glutamate was suggested by Kahn *et al.* (6). More recently, Kohl *et al.* (9) reported high proline synthetic activity in plant cytosol and high proline oxidizing activity in bacteroids from soybean nodules, and put forward the hypothesis that at least some of the energy required for N_2-fixation is supplied to the bacteroid in the form of proline. Both glutamate (14) and proline (our unpublished results) can stimulate N_2-fixation by anaerobically isolated soybean bacteroids. ^{15}N enrichment of bacteroids might result if a significant quantity of glutamate (either supplied directly to the bacteroid or produced by oxidation of proline) is deaminated within the bacteroid forming oxoglutarate, which can enter the tricarboxylic acid cycle to provide energy for nitrogenase activity. While the isotope effect for the deamination of glutamate has not been measured, to the degree to which it is expressed in this system, unreacted glutamate would be enriched in ^{15}N. If the predominant portion of the glutamate pool is deaminated, then the minor quantity incorporated into bacteroid protein (as glutamate or other amino acids derived from its amino N) could be considerably enriched in ^{15}N. If substantial quantities of glutamine were imported, as suggested by the presence of high glutamate synthase and low glutamine synthetase activity in soybean bacteroids (2), the result would be essentially the same. The lack of ^{15}N enrichment of bacteroids from peas might reflect a difference in energy source (*e.g.* relatively more organic acids compared to amino acids). At the present time we can offer no explanation for the substantial ^{15}N enrichment of soybean nodule cortex.

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